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## Physiological responses of the fan mussel *Pinna nobilis* to temperature: ecological and captivity implications

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### Abstract

The fan mussel *Pinna nobilis* is experiencing a mass mortality event throughout the Mediterranean Sea. The survival of the remaining isolated populations and the maintenance and reproduction of individuals in captivity could determine the future of the species. This paper examines the clearance rate (CR) and oxygen consumption ( $VO_2$ ) of fan mussel individuals measured under 5 different temperatures (8.5, 14, 18, 23 and 28°C). The measurements of both physiological variables revealed a threshold limit of thermal tolerance at both extreme temperatures, indicating the critical situation of the remaining populations, which are located in coastal lagoons and shallow bays where these extreme temperatures usually occur. Besides, the high clearance rates observed highlight the significant ecosystem service provided by the species in terms of water filtration, especially in confined waters with low renewal rates. Routine control of the clearance rate as an early warning system is proposed for detecting ailing individuals. Such a system could also be used to improve captivity conditions.

**Keywords:** physiology; fan mussel; invertebrate welfare; climatic change; ecosystem services.

### Introduction

The fan mussel *Pinna nobilis* is an endangered species threatened with extinction (Kersting *et al.*, 2019) by a disease caused by *Haplosporidium pinnae* (Panarrese *et al.*, 2019; Grau *et al.*, 2022), sometimes together with a *Mycobacterium* sp. and other pathogens (Carella *et al.*, 2020; Lattos *et al.*, 2021a; Lattos *et al.*, 2021b). The die-off, which started in late 2016 (Vázquez-Luis *et al.*, 2017), has provoked high mortalities, close to 100% of individuals throughout the Mediterranean Sea (Acarli *et al.*, 2020; Öndes *et al.*, 2020; Özalp & Kersting, 2020; Šarić *et al.*, 2020; Betti *et al.*, 2021; Katsanevakis *et al.*, 2022). Few populations remain unaffected or partially affected in paralic environments, the only reservoirs of fan mussel populations known to date (García-March *et al.*, 2020). In the Mediterranean Sea, the effects of cli-

matic impacts are expected to be particularly exacerbated in these environments, due to oceanographic conditions such as restricted circulation, shallow depth, and large water residence (Kennish & Paerl, 2010). Mean global warming is expected to reach 1.5°C during the following three decades (IPCC, 2018) exerting a negative effect on marine biodiversity (Jones & Cheung, 2015). The Mediterranean Sea is particularly sensitive to climate warming (Giorgi & Lionello, 2006) with heatwaves increasing in duration and intensity (Molina *et al.*, 2020; Garrabou *et al.*, 2022) and causing mortalities of marine benthic invertebrates (Garrabou *et al.*, 2009; Rivetti *et al.*, 2014; Garrabou *et al.*, 2019; Garrabou *et al.*, 2022).

The few fan mussel populations that can still be found in shallow bays, coastal lagoons and the Marmara Sea (García-March *et al.*, 2020; Cinar *et al.*, 2021; Nebot-Colomer *et al.*, 2021; Peyran *et al.*, 2021; Acarli *et al.*, 2022;

Katsanevakis *et al.*, 2022) are threatened in the medium to long-term (García-March *et al.*, 2019), but information on how resilient can they be to expected changes is lacking. Moreover, the connection between surviving populations and impacted areas (Wesselmann *et al.*, 2018; González-Wangüemert *et al.*, 2019) could be pointless because of the persistence of the disease (protozoan spores), which prevents recolonization by propagule rain after local extinction. Thus, the importance of preserving the few remaining unaffected populations is significant. However, the presence of resistant juveniles has been reported and this is promising as regards the long-term survival of the species (Ruitton & Lefebvre, 2021), coupled with the isolated recruitment events at a few locations recorded by Kersting *et al.* (2020)

The functional alterations of the possible disappearance of an endemic species such as *P. nobilis* are also uncertain, but the loss of biodiversity can alter the capacity of water ecosystems to provide food, maintain their water quality and recover from perturbations (Worm *et al.*, 2006). In fact, one of the main roles associated with *P. nobilis* is its contribution to water clarity retaining large amounts of organic matter (Trigos *et al.*, 2014), presumably by filtering large amounts of seawater, although their real capacity is still unknown. Besides, the fan mussel contributes to the increase of biodiversity by generating hard substrate that is used by other species to settle (Kuhlmann, 1998; Cosentino & Giacobbe, 2008; Rabaoui *et al.*, 2009).

Physiological measurements of clearance rate (CR) in bivalves, as the volume of water filtered and cleared of particles, can be used as a biomarker of individual conditions (Toro *et al.*, 2003) and is also the main physiological variable determining the scope for growth (SFG), representing the energy available for somatic and gonadal growth (Beiras *et al.*, 1994; Albentosa *et al.*, 2012b). Besides, CR exemplifies the role of bivalves in the ecosystem acting as biofilters and retaining nutrients (Dame, 1993; Odd *et al.*, 2005; Rose *et al.*, 2015). The measurement of oxygen consumption ( $VO_2$ ) is also considered of great importance and is used to estimate metabolic expenses incurred for maintaining vital functions (Bayne & Newell, 1983), and to approximate maintenance rations required to fulfil energy requirements (Albentosa *et al.*, 2012a). Both physiological functions, CR and  $VO_2$ , are greatly influenced by temperature (Griffiths & Griffiths, 1987), whose effect in the context of global warming could define the future of the species. In fact, Basso *et al.* (2015) detected higher mortality rates of individuals at 26°C compared to lower temperatures. Moreover, lower life expectancy has been observed in populations inhabiting paralic environments, indicating that more extreme environmental conditions might be one of the possible reasons (García-March *et al.*, 2019). Moreover, temperature is a key factor for gonad maturation during the reproductive period (Utting & Millican, 1997) and related to recruitment success (Kersting & García-March, 2017), but also considered a key factor controlling the disease caused by *H. pinnae*, which showed a seasonal pattern and higher expression at temperatures above 13.5°C (Ca-

banellas-Reboredo *et al.*, 2019). Individuals affected by *H. pinnae* resisted longer under lower temperatures in captivity (García-March *et al.*, 2020), although the capacity to overcome the disease is unclear, but the use of low temperatures in captivity at the beginning of the quarantine period could help in reducing the transmission of the disease and isolate sick individuals.

In this work, CR and  $VO_2$  were measured in *P. nobilis* under five different temperatures. Knowledge of basic physiological parameters such as CR and  $VO_2$  and their variations with temperature could help to improve captivity protocols and quantify the filtration capacity and energy requirements of fan mussels. Besides, it will enhance our general understanding of ecosystem services provided by fan mussels and the vulnerability of the species, especially in the context of the populations located in paralic environments, and the consequences of climate change in the Mediterranean Sea (Difffenbaugh *et al.*, 2007).

## Material and Methods

### Collection and acclimation of individuals

Eight *P. nobilis* individuals of similar size ( $\approx 30$  cm) were collected in February 2020, from Fangar Bay, in the Ebro Delta (Catalonia, NW Mediterranean). Individuals were transported fully submerged in aerated coolers to the IMEDMAR-UCV facilities in Calpe (Spain). Once in the lab, they were measured in length and width and kept for 6 months in two closed 750 L tanks, each with a closed circuit. Infection by *H. pinnae* was untested, given the health condition of the Fangar Bay population at the moment, although individuals were daily checked for symptoms of disease such as mantle retraction and lack of response (García-March *et al.*, 2020). The individuals were placed in vertical position, with one third of the shell buried in coral debris substratum within individual plastic buckets. Water was maintained at  $14.0 \pm 0.5^\circ\text{C}$  and salinity at 37.5 – 38.0 psu. During this period and during the thermal experiment, the fan mussels were continuously fed daily 16h a day with the filters deactivated. Their diet was composed by a mix of live *Isochrysis galbana* (T-ISO) and *Chaetoceros calcitrans* cultured *in situ* at a ratio of 50-80% and 50-20% respectively, depending on culture conditions. Each day, an initial dose of  $\approx 2 \text{ mm}^3 \text{ L}^{-1}$  ( $\approx 45,000 \text{ cel ml}^{-1}$ , concentration below the pseudofaeces threshold) was provided (Bayne *et al.*, 1989; Bayne, 1993). Later, half of the initial dose was given again when the remaining concentration of microalgae reached 50% of the initial dose. During the following 8h, no food was pumped into the aquariums and the filters were activated to allow water cleaning until the next day.

### Experimental design

For thermal experiments, the 8 specimens of *P. nobilis* were individually confined in eight equal 55 L aquariums. One extra aquarium without an individual was used

as control with the same water conditions to correct the sedimentation rate of phytoplanktonic particles in the experimental aquariums. The water was circulated from the aquaria through a sump equipped with a mechanic and biological filter, a skimmer, and a temperature regulation system and then returned to the aquaria. The fan mussels were placed in horizontal position, as no significant differences in the physiology of Pinnids were observed between laying down and standing positions (Hernandis *et al.*, 2022).

In order to analyse the effects of temperature on *P. nobilis*, clearance rates (CR: L h<sup>-1</sup>) and oxygen consumptions (VO<sub>2</sub>: mg O<sub>2</sub> h<sup>-1</sup>) at 5 temperatures were tested: 8.5, 14, 18, 23 and 28 ± 0.5°C. The lowest temperature (8.5°C) was considered the coldest temperature that can occur in shallow Mediterranean areas such as coastal lagoons (Amos *et al.*, 2017; Marques *et al.*, 2019; Tagliapietra *et al.*, 2021). A water temperature of 14°C is common in winter in the Mediterranean Sea (García-March *et al.*, 2011), and 18 and 23°C represent intermediate temperatures within the thermal comfort range of the species (Basso *et al.*, 2015; Trigos *et al.*, 2015). Finally, 28°C was considered as the highest temperature in the experiment for testing the upper thermal limit of the species. Similar temperatures can be observed in Fangar Bay, from where the specimens were collected, although a wider range of temperatures may occur, reaching a minimum of 6°C and a maximum above 30°C (Ramón *et al.*, 2007). However, given the protected status of the species, these extreme temperatures were avoided in order to reduce the risk of mortality due to experimental stress or excessive bacterial growth. The experiment was performed according to the following thermal-change protocol: initially, water temperature was decreased from 14°C (acclimation temperature) to 8.5 (1 degree day<sup>-1</sup>). Subsequently, the individuals were kept at this temperature for 1 week and CR and VO<sub>2</sub> were measured on the 7<sup>th</sup> day. Once measurements were completed, water temperature was increased to the next exposure-temperature (1 degree day<sup>-1</sup>), and the physiological measurements were determined after 7 days.

After completion of the experiment, the individuals were kept in captivity for further experiments.

### Physiological measurements

#### Clearance rate (CR: L g<sup>-1</sup> h<sup>-1</sup>)

Seawater recirculation in the aquariums was interrupted to measure clearance rates. Subsequently, a food dose (≈ 46816 cel ml<sup>-1</sup>, ≈ 2.12 mm<sup>3</sup> L<sup>-1</sup>, POM ≈ 1.14 mg MO L<sup>-1</sup>) of *Isochrysis galbana* (T-ISO) was added to each aquarium and the clearance rate was calculated from the depletion of particle concentration in the aquarium following the expression of Coughlan (1969):

$$\ln(C_0) - \ln(C_t) = \left(\frac{CR}{V} + \alpha\right) \cdot t$$

Where C<sub>0</sub> and C<sub>t</sub> represent particle concentration at time 0 and t, respectively, V is the water volume in the aquarium (55 L) and α is the particle sedimentation rate (deter-

mined as Ln C<sub>0</sub> - Ln C<sub>t</sub> = α t in a control aquarium without animal).

Particle concentration was determined using an electronic particle counter, Coulter-Multisizer III, with an aperture tube of 100 μm. The first sample (C<sub>0</sub>) was taken immediately after the addition of the microalgae once mixed. Later, 5 more samples were taken at regular intervals, 7 to 25 min intervals, depending on the temperature (the CR at low temperatures was lower; therefore, the time between samples was higher to detect differences in microalgae concentration). This procedure was repeated four consecutive times for each individual and exposure temperature. To clean any remains of phytoplankton between replicates, water was recirculated through the system filter and through a 1 μm filter. Finally, individual clearance rate was computed as the mean CR value obtained for each individual using the 4 replicates.

#### Oxygen consumption (VO<sub>2</sub>: mg O<sub>2</sub> g<sup>-1</sup> h<sup>-1</sup>)

Once CR measurements were completed, the fan mussels were transferred to respirometers in order to determine consumption. Respirometers consisted of 15L plastic buckets, sealed and filled with water at the experimental temperature. Oxygen consumption was computed as the rate of reduction in dissolved oxygen concentration (DOC) in the respirometer over time. DOC was measured every 15 min (30 min at 8.5°C) using the oxygen sensor of the oceanographic probe AAQ-RINKO 177 and a circulation pump to allow water movement and guarantee correct functioning of the oxygen sensor.

Individuals were kept in the bucket until a 20% difference in oxygen concentration was detected, or a maximum of 3 hours had passed. Given the initial stress caused by handling, measurements were initiated 15 min after placing the individuals in the bucket. To maintain a constant temperature, respirometers were placed inside a 350L tank at the experimental temperature and with climate control.

#### Expression of Physiological rates

Physiological rates are usually standardized to 1g of meat dry weight (DW) using allometric coefficients to correct the inter-individual differences in size. However, since no allometric studies of the physiological variables are currently available for this species, we could not proceed to standardized physiological rates. Besides, the dissection of experimental animals was avoided given the endangered status and the critical situation of the species. Hence, the dry weight of the soft tissues of the experimental specimens was estimated using the length-dry weight regression available for *P. rudis*: (R<sup>2</sup> = 0.97, p < 0.001) the closest species for which published data is available (Hernandis *et al.*, 2021). Therefore, physiological rates were expressed as mass-specific rates by dividing individual physiological rates by the estimated dry weight of soft-tissues.

Thermal dependence of physiological variables was analysed by computing  $Q_{10}$  coefficients, using the equation where  $R_2$  and  $R_1$  are the physiological rates at temperature  $t_2$  and  $t_1$  respectively (Bayne & Newell, 1983). The  $Q_{10}$  coefficient represents the dependency of an organism's physiology on temperature changes and varies according to the thermal history of the animal, body size, activity, and reproductive conditions (Gosling, 2015).  $Q_{10}$  values near 1 indicate thermal independency while a value of 2 represents doubling of the physiological rate with each 10°C increase in temperature. In general,  $Q_{10}$  values between 1 and 3 indicate non-stressful conditions whereas values > 4 indicate thermal stress (Zippay & Helmuth, 2012).

The ratio between CR and  $VO_2$  was also calculated to check for variations in the physiological performance of both processes.

### Statistical analysis

An analysis of variance (ANOVA) was used to test for differences in CR,  $VO_2$  and the rate of both variables between temperatures. A one-way factor RM-ANOVA was applied with "temperature" as a fixed factor with five levels: 8.5, 14, 18, 23 and 28°C and a pair-wise t-test with paired groups was carried out to detect differences among them. Before each analysis, the Shapiro-Wilk test was used to check if data followed a normal distribution. All statistical analyses were performed using R statistical computing environment, and the results are expressed as mean  $\pm$  standard error (mean  $\pm$  SE).

## Results

### Biometric data

The mean size of individuals was  $29.4 \pm 2.2$  cm of shell length and  $12.0 \pm 1.0$  cm of shell width. The estimated mean DW was  $5.7 \pm 1.0$  g (using the length-dry weight regression described by Hernandis *et al.* (2021) for *Pinna rudis*). The biometric data for each individual is presented in Table 1.

**Table 1.** Biometric data for each *Pinna nobilis* individual.

Individual	Length (cm)	Width (cm)	DW* (g)
1	29.30	11.45	5.62
2	32.20	13.20	7.09
3	31.40	12.00	6.67
4	31.10	12.40	6.51
5	29.40	11.30	5.66
6	26.50	12.00	4.38
7	26.20	10.40	4.26
8	29.10	13.20	5.52

\*Estimated dry weight obtained using the length-dry weight regression available for *Pinna rudis* (Hernandis *et al.*, 2021).

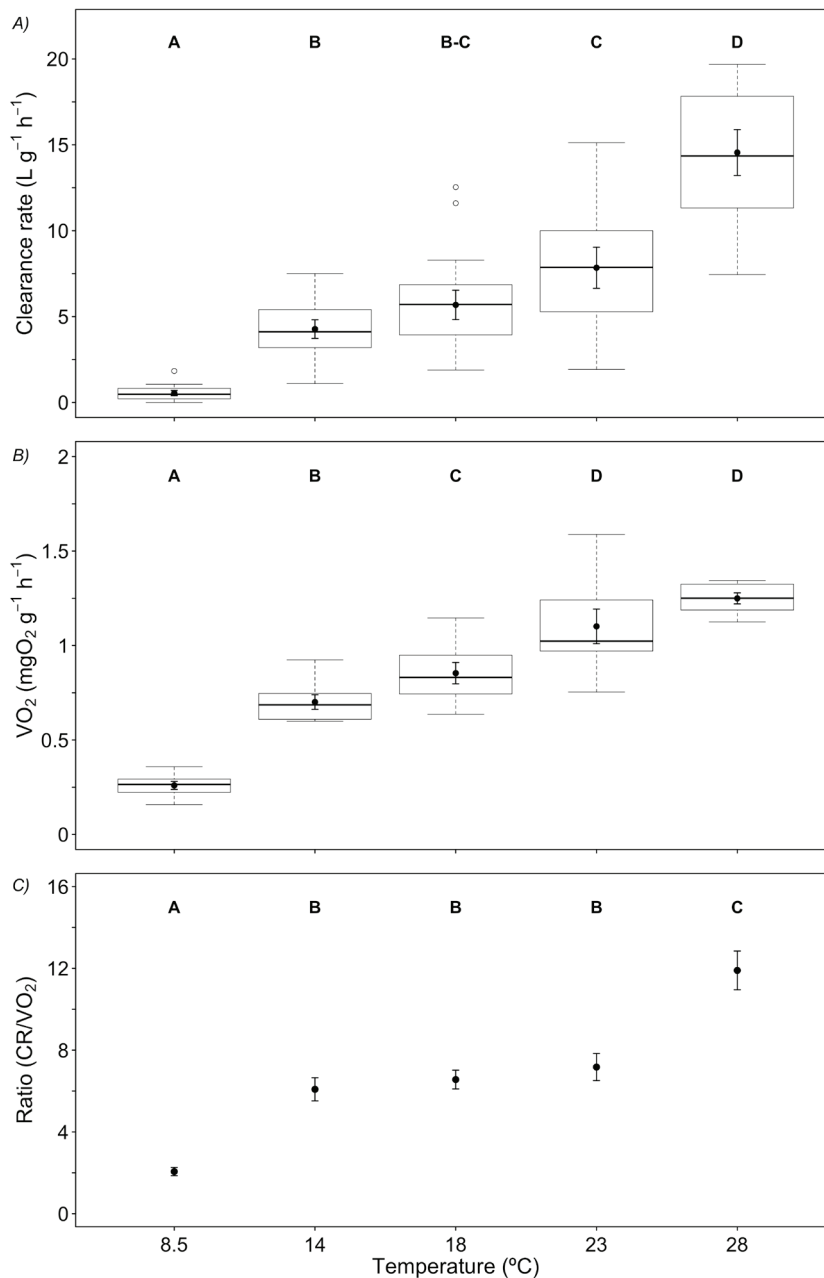
### Physiological measurements

Mean clearance rate (CR:  $L g^{-1} h^{-1}$ ) of fan mussels increased sharply with water temperature (one-way ANOVA,  $F = 38.17$ ,  $p < 0.01$ , Fig. 1A). In the comparison between temperatures, the pair-wise t-test showed that in the cold temperature range (8.5 to 14°C) mean CR varied from  $0.55 \pm 0.15$  to  $4.27 \pm 0.54 L g^{-1} h^{-1}$ ,  $p < 0.01$  and  $Q_{10} = 56.59 \pm 16.75$  (Table 2). In the mild temperature range (14 to 23 °C), the clearance rate increased to  $5.68 \pm 0.86$  at 18°C and to  $7.84 \pm 1.20 L g^{-1} h^{-1}$  at 23°C ( $Q_{10}$  approximately 2, Table 2), with only significant differences ( $p < 0.05$ ) between 14 and 23°C. Finally, the thermal sensitivity of the clearance rate increased significantly in the warmest range (23 to 28°C): CR rose significantly from  $7.86 \pm 1.20$  to  $14.55 \pm 1.34 L g^{-1} h^{-1}$  for 6 individuals,  $p < 0.05$  and  $Q_{10} = 6.03 \pm 2.64$  (Table 2). The other two maintained their valves almost closed during the day of CR measurements and showed a reduction of their filtration below  $4 L g^{-1} h^{-1}$ .

For 28°C, a spawning event was observed before the experiment, where all individuals released gametes (7 males and 1 female). The experiment was postponed for 48h to allow the individuals to recover.

Mean values of mass-specific oxygen consumption ( $VO_2$ :  $mg O_2 g^{-1} h^{-1}$ ) also increased significantly with temperature (one-way ANOVA,  $F = 111.00$ ,  $p < 0.01$ , Fig. 1B). In the comparison between temperatures, the pair-wise t-test showed that in the cold temperature range (8.5 to 14°C),  $VO_2$  evidenced high thermal dependency ( $Q_{10} = 6.60 \pm 1.00$ , Table 2), significantly increasing from values of  $0.26 \pm 0.02$  to  $0.70 \pm 0.04 mg O_2 g^{-1} h^{-1}$  ( $p < 0.01$ ). At mild temperatures (14 to 23°C),  $VO_2$  showed relatively reduced thermal dependence ( $Q_{10}$  of approximately 1.7, Table 2) significantly increasing to  $0.85 \pm 0.06 mg O_2 g^{-1} h^{-1}$  at 18°C and to  $1.10 \pm 0.09 mg O_2 g^{-1} h^{-1}$  at 23°C ( $p < 0.05$ ). Contrary to the trend displayed by CR, the  $VO_2$  of fan mussels did not significantly increase in the upper thermal range (23 to 28°C), which varied from  $1.10 \pm 0.09 mg O_2 g^{-1} h^{-1}$  at 23°C to  $1.25 \pm 0.03 mg O_2 g^{-1} h^{-1}$  at 28°C ( $p > 0.05$ ).

Significant differences were observed in the CR/ $VO_2$  ratio (Fig. 1C) (one-way ANOVA,  $F = 25.13$ ,  $p < 0.01$ ). In the comparison between temperatures, the pair-wise



**Fig. 1:** Mean values  $\pm$  standard error of: A) Mass-specific clearance rates (CR:  $L\ g^{-1}\ h^{-1}$ ) of *Pinna nobilis* individuals in relation to water temperature ( $n = 8$ , at  $28\ ^\circ C\ n = 6$ ). B) Mass-specific oxygen consumption ( $VO_2$ ;  $mg\ O_2\ g^{-1}\ h^{-1}$ ) of *P. nobilis* individuals in relation to water temperature ( $n = 8$ ). C)  $CR/VO_2$  ratios at the different experimental temperatures for *P. nobilis* individuals ( $n = 8$ ). Letters A - D represent statistical different groups ( $n = 6$ ). A black point and solid vertical lines indicate mean and standard error respectively.

**Table 2.**  $Q_{10}$  coefficient  $\pm$  standard error (SE) results for physiological measurements of clearance rate (CR) and oxygen consumption ( $VO_2$ ) in *Pinna nobilis* individuals.

Temperature ( $^\circ C$ )	CR $Q_{10} \pm SE$	$VO_2$ $Q_{10} \pm SE$
8.5 — 14	$56.59 \pm 16.75$	$6.60 \pm 1.00$
14 — 18	$2.68 \pm 0.59$	$1.67 \pm 0.18$
18 — 23	$2.20 \pm 0.36$	$1.72 \pm 0.23$
23 — 28	$6.03 \pm 2.64$	$1.45 \pm 0.24^*$

\*No significant differences were found in oxygen consumption ( $VO_2$ ) within the range  $23\text{--}28\ ^\circ C$ , therefore the  $Q_{10}$  between these temperatures could be considered as 1.

t-test did not reveal significant differences ( $p > 0.05$ ) in the comparison at mid temperatures (14, 18 and 23°C), where a relatively constant value of  $\approx 6.5$  was observed. However, significant differences were observed ( $p < 0.01$ ) compared to both 8.5 and 28°C, where the ratio decreased to 2.1 and increased to 11.9, respectively.

## Discussion

### *Thermal tolerance and physiological measurements*

Temperature is one of the main factors affecting metabolism in bivalves (Newell & Branch, 1980) and thus influencing their filtration capacity (Fuchs & Specht, 2018; Specht & Fuchs, 2018). Clearance rate (CR) typically increases with temperature up to the limit of tolerance for the species when a rapid decline is observed (Griffiths & Griffiths, 1987). Accordingly, in this study, the CR of *P. nobilis* increased with temperature, reaching a maximum at 28°C. The mass-specific CRs in *P. nobilis* recorded during this study (from a mean of 0.55 to 14.55 L g<sup>-1</sup> h<sup>-1</sup>) were higher than in other bivalve groups such as mussels, scallops, oysters, and cockles (from 2.47 to 3.57 L g<sup>-1</sup> h<sup>-1</sup> according to the meta-analysis performed by Cranford *et al.* (2011)). Yet, higher CRs of 25.88 L g<sup>-1</sup> h<sup>-1</sup> at 28°C have been reported for *Pinctada margaritifera* (Pouvreau *et al.*, 1999). In the context of other members of the Pinnidae, the CR recorded at 18°C in this study (5.68 L g<sup>-1</sup> h<sup>-1</sup>) was similar to that indicated by Hernandis *et al.* (2022) for *P. rudis* (5.29 L g<sup>-1</sup> h<sup>-1</sup>) at that temperature. However, Nieves-Soto *et al.* (2013) observed much lower CRs at all temperatures in *Atrina tuberculosa* (0.37, 1.39, and 3.96 L g<sup>-1</sup> h<sup>-1</sup> respectively at 17, 22.5 and 28°C) compared to *P. nobilis* in the present study (5.68, 7.86 and 14.55 L g<sup>-1</sup> h<sup>-1</sup> respectively at 18, 23 and 28°C), which could be related to methodological differences in the flow-through system used to measure CR.

The effect of temperature on the metabolism and CR of bivalves is directly associated with VO<sub>2</sub> (Buxton *et al.*, 1981) because of the higher energetic expenditure with increased CR (Navarro *et al.*, 1992; Velasco & Navarro, 2002). Accordingly, an increase of VO<sub>2</sub> with temperature was observed in *P. nobilis* over the entire investigated range (8.5-23°C), although no significant differences were detected from 23 to 28°C. However, Trigos *et al.* (2015) observed a higher VO<sub>2</sub> at 20°C than at 25°C. A possible explanation could be that different individuals were used for experimental conditions at 20 and 25°C, and that those results were given per individual, not in standardized values. In fact, they observed lower values of oxygen consumption per individual at 16°C (3.1 mg O<sub>2</sub> ind<sup>-1</sup> h<sup>-1</sup>) compared to those found in this study at 14°C (4.2 mg O<sub>2</sub> ind<sup>-1</sup> h<sup>-1</sup>, equivalent to 0.70 mg O<sub>2</sub> g<sup>-1</sup> h<sup>-1</sup>) even though the individuals of this study were smaller and the temperature was lower. On the contrary, Basso *et al.* (2015) observed a much higher VO<sub>2</sub> compared to that of this study, with values above 1.5 mg O<sub>2</sub> g<sup>-1</sup> h<sup>-1</sup> at 20 and 23°C, and over 3.0 mg O<sub>2</sub> g<sup>-1</sup> h<sup>-1</sup> at 26°C compared to values of 1.25 mg O<sub>2</sub> g<sup>-1</sup> h<sup>-1</sup> in this study at 28°C. The var-

iations between Basso *et al.* (2015) and our study could be explained by differences in size. The individuals used by Basso *et al.* (2015) were smaller (maximum length of 9.48cm) than those of our study, and a higher mass-specific oxygen consumption can be expected in smaller individuals (Bayne & Newell, 1983). Alternatively, differences in VO<sub>2</sub> could be due to variations in the thermal optimum induced by differences in acclimation temperature (Newell *et al.*, 1977; Buxton *et al.*, 1981), which was 20°C in Basso *et al.* (2015) vs. 14°C in our work. Each of these arguments agrees with the results observed by Hernandis *et al.* (2022) in *P. rudis*; the authors found that medium-sized individuals (18.4 cm), acclimated at 18°C, showed an intermediate VO<sub>2</sub> (1.61 mg O<sub>2</sub> g<sup>-1</sup> h<sup>-1</sup> at 18°C in *P. rudis* vs. 0.92 mg O<sub>2</sub> g<sup>-1</sup> h<sup>-1</sup> at 18°C in this study and 1.55 – 3.51 mg O<sub>2</sub> g<sup>-1</sup> h<sup>-1</sup> at 20°C in Basso *et al.* 2015a).

The Q<sub>10</sub> values of fan mussels were around 2 for both, CR and VO<sub>2</sub>, over intermediate temperature ranges (14-18°C and 18-23°C), thus indicating thermal dependency but non-stressful conditions for both variables. Similar Q<sub>10</sub> values for CR and VO<sub>2</sub> have been obtained for other bivalves at comparable temperatures. In *Mytilus* spp. Q<sub>10</sub> values below 3 have been obtained with acclimatization temperatures ranging from 10 to 22°C (Widdows, 1973; Van Erkom Schurink & Griffiths, 1992; Barbariol & Razouls, 2000), indicating, occasionally, thermal independence (Q<sub>10</sub>  $\approx$  1) that has been associated with individuals living in intertidal conditions (Eckert *et al.*, 1988). Furthermore, for *Ostrea* spp. Q<sub>10</sub> values near 2 have been observed at temperatures from 10 to 23°C (Rodhouse & O'Kelly, 1981; Sytnik & Zolotnitskiy, 2014). Over more extreme temperature ranges, fan mussels showed higher Q<sub>10</sub> values, indicating stressful conditions at 8.5 and 28°C, as observed for other bivalve species such as *Pinctada mazatlanica* and *Venerupis pullastra* at similar temperatures (Albentosa *et al.*, 1994; Saucedo *et al.*, 2004) with Q<sub>10</sub> values > 4 that indicate thermal dependence (Zippay & Helmuth, 2012), and suppression of CR at 8.5°C was observed. This suppression of CR at 8.5°C could explain the extremely high Q<sub>10</sub> of 56.59 observed over the 8.5- 14°C range. It should be noted that individuals were acclimated at 14°C during a 6- month period; this could have affected optimum thermal performance and, therefore show a better fit at 14°C than at other temperatures (Bayne, 2017). From 14 to 23°C, the CR/VO<sub>2</sub> ratio showed low variability with the relationship between the two variables remaining close to a flat line. However, bigger differences were observed for this ratio at 8.5 and 28°C. CR almost doubled from 23 to 28°C while no significant increase in VO<sub>2</sub> was observed, thus suggesting the implication of anaerobic metabolism for maintaining the energy expenses of this higher filtration activity. Thermal tolerance limits are defined by the upper and lower “pejus temperature” – the limit of optimum hemolymph oxygenation, where processes such as growth and reproduction are negatively affected– (Pörtner, 2002). Beyond those limits, the aerobic scope is reduced toward the upper and lower “critical temperatures”, where the occurrence of anaerobic metabolism is necessary to meet the energy metabolism demand (Bayne, 2017). In this ex-

periment, the high increase of CR but not  $VO_2$  at 28°C suggests the implication of anaerobic metabolism in supplying the extra energy required; thus, the critical temperature would have been exceeded. In marine invertebrates, anaerobic metabolism in oxygenated waters could imply approach to the threshold thermal tolerance limit (Anestis *et al.*, 2007) with the resistance of individuals becoming a matter of time depending on their starvation tolerance (Pörtner, 2002). Response to extreme temperatures can vary significantly between individuals (Bayne, 2017) and it was observed that two fan mussels drastically reduced their filtration activity, which is considered detrimental (Williams *et al.*, 2016; Bayne, 2017). Similarly, Basso *et al.* (2015) observed thermal stress at 26°C with higher mortality in stabled *P. nobilis* juveniles compared to lower temperatures of 20 and 23°C during long-term exposure (36 days). Prado *et al.* (2019) also observed high mortality of fan mussel individuals when temperatures were above 25°C, which triggered infection by *Vibrio mediterranei*. At low temperatures (8.5°C), a clear decrease in CR and  $VO_2$  was observed. The lower CR/ $VO_2$  ratio at this temperature suggests low gill activity, which could cause starvation and low oxygen uptake (Pörtner, 2002), indicating stressful conditions for the species. In both scenarios, 8.5 and 28°C, a catabolic process could be playing a major role as a response to insufficient energy obtained through respiration, as previously observed for *P. nobilis* individuals under inadequate diets (Prado *et al.*, 2021a). It is important to consider that the inherent stress of captivity could make *P. nobilis* even more vulnerable to high temperatures and associated diseases compared to individuals in the open sea (Dickens *et al.*, 2010). However, no mortality was observed in our experiment. The shorter exposure to each extreme temperature (one week) was probably too short to cause excessive pathogen growth or stress, leading to mortality.

Bivalves are able to acclimate to temperature variations; they usually display short-term response – acute response – in the following hours-days (few hours - 2 days) (Cusson *et al.*, 2005; Peteiro *et al.*, 2018). Subsequently, the compensation process allows the individuals to acclimate to the new temperature, which starts after  $\approx 3$  days (Cusson *et al.*, 2005) and requires several days to achieve ( $\approx 7$  to 21 days) (Bayne, 2017; Peteiro *et al.*, 2018). This acclimation allows to extend the temperature range over which a positive energy balance is achieved. In this sense, Beiras *et al.* (1995) observed that oyster spat acclimated at 20°C showed a decrease in CR after 1 day at 14°C. However, after 8 and 16 days at this temperature, the individuals were able to compensate the thermal change partially by increasing CR and slightly decreasing the metabolic cost ( $Q_{10}$  of 3.55, 2.50, 1.68 respectively for day 1, 8 and 16). Nevertheless, there are limits to acclimation (Bayne, 2017). In our work, one week was considered sufficient to compensate each temperature, almost entirely. An extension of the exposure time would have involved a higher and unnecessary risk for the individuals given the species status. Besides, after one week at each temperature, high  $Q_{10}$  ( $> 6$ ) values were still observed at 8.5°C (for CR and  $VO_2$ ) and 28°C (for CR), presumably indicating that fan

mussels were hardly able to further physiologically compensate at these temperatures. Furthermore, the results obtained are conservative considering that more extreme temperatures can be found in the paralic environments where the remaining populations survive, over a temperature range that could reach limits as low as  $\approx 6^\circ\text{C}$  during winter, and above 30°C in summer (Pérez-Ruzafa *et al.*, 2005; Ramón *et al.*, 2007; Amos *et al.*, 2017; Marques *et al.*, 2019; Tagliapietra *et al.*, 2021); therefore, harder conditions for the fan mussel can be expected at those sites.

It is also important to note that individuals could have experienced higher stress compared to that under natural conditions, as regards the speed of increase in temperatures during the experiment. However, 8.5°C was the first temperature tested after a long acclimation period to 14°C; therefore, the accumulated stress at this temperature was lower. As for 28°C, despite the faster increase in temperatures compared to natural conditions, normal physiological results ( $Q_{10}$ ) were recorded at intermediate temperatures (14, 18 and 23°C), with physiological results comparable to those other bivalve species such as *P. rudis* (Hernandis *et al.*, 2022). Although, additional stress due to the experimental design was expected, the main effect could be associated with exposure to extreme temperatures.

Regarding the spawning event observed in all individuals, it could be associated with stress conditions and triggered by the high temperatures (Barber & Blake, 2006). It is relevant to mention that, given the current situation of the species, captivity reproduction should be a priority for the fan mussel, although this is beyond the scope of the current work.

The high mass-specific CR observed in *P. nobilis* coupled with its large size confirms the assumption that fan mussels are outstanding water filters. Besides, if we consider that the species can reach up to 120cm (Zavodnik *et al.*, 1991) in length and that lengths of over 50cm are commonly observed (García-March *et al.*, 2019), higher CR per individual can be expected for much larger adults than those used in this study ( $\approx 30$ cm of shell length). The environmental impact in terms of water filtration of fan mussels would be substantial, especially for large populations in confined waters which, in addition, are the only areas with individuals enduring the disease, like the Mar Menor lagoon (Nebot-Colomer *et al.*, 2021; Cortés-Melendreras *et al.*, 2022), the Ebro Delta (Prado *et al.*, 2021b), several coastal lagoons in the Gulf of Lion (Peyran *et al.*, 2021), the Marano lagoon (Curiel *et al.*, 2020) and the Sea of Marmara (Acarli *et al.*, 2022). These paralic environments are particularly susceptible to eutrophication because of high anthropogenic impacts and lower water renewal compared to the open sea (Kennish & Paerl, 2010); therefore, the relevance of fan mussel water filtration is greater. In Alfacs Bay, where the fan mussels were collected for this study, the estimated population in 2011-2012 was 90,303 individuals (Prado *et al.*, 2014). The bay receives nutrients from agriculture irrigation (Mañosa *et al.*, 2001; Falco *et al.*, 2010), has an approximate volume of 150 hm<sup>3</sup> (Jordana *et al.*, 2015) and the water renewal time is 45 days in the inner parts of the



bay, decreasing considerably near the mouth (Cerralbo *et al.*, 2019). Considering the observed CR, we roughly estimate that 2.06% of the Alfacs Bay volume would have been filtered in a month at 23°C.

For the Mar Menor, the largest saltwater lagoon in Europe (598.8 hm<sup>3</sup>), the water filtration service provided by fan mussels would have been higher (up to ca. 10% of the lagoon volume in a month at 23°C) due to the lower water renewal of 384 days (Umgiesser *et al.*, 2014) and the much larger population reported, with up to 1.6 million of estimated individuals in 2013-2014 (Giménez-Casaldueiro *et al.*, 2020). However, the threat of eutrophication is constant (Velasco *et al.*, 2006; Robledano Aymerich *et al.*, 2008) and caused the collapse of the lagoon in late 2015 (García-Ayllon, 2018) resulting in the loss of over 99% of the population (Nebot-Colomer *et al.*, 2021; Cortés-Melendreras *et al.*, 2022).

Unfortunately, these enclosed areas are more prone to extreme temperatures than the open sea because of the prevailing oceanographical conditions (Quintana *et al.*, 2018). In summer, the water temperature in these areas can exceed the 28°C, while temperatures as low as 8°C are also commonly reported during the winter (Yalçın *et al.*, 2017; Marques *et al.*, 2019; Romero *et al.*, 2020; Llorca *et al.*, 2021; Prado *et al.*, 2021b; Tagliapietra *et al.*, 2021). In fact, the harsh conditions of these areas have been pointed out as one of the reasons of the lower life expectancy of fan mussel individuals inhabiting them (García-March *et al.*, 2019). Overreaching the thermal comfort at 8.5 and 28°C could increase the impact of additional environmental pressures, and highlight the critical situation of the species in paralic environments, the remaining reservoirs. All this taking place in a context of climate change, increased heatwaves, and weather extreme conditions in duration and intensity (Cramer *et al.*, 2018; IPCC, 2018; Abbasnia & Toros, 2019), where these environments will be especially affected. These sites are of high importance for the natural recovery of the species through larval export (Kersting *et al.*, 2020) which could be also negatively affected by these stressful conditions.

### **Implications for *Pinna nobilis* in captivity**

The die-off affecting *P. nobilis* has been associated with *H. pinnae* at temperatures above 13.5°C (Cabanelas-Reboredo *et al.*, 2019; García-March *et al.*, 2020), similar to the mortality of captive individuals by *Vibrio* infection at temperatures >17°C (peaked at 25°C) (Prado *et al.*, 2019; Andree *et al.*, 2020; Prado *et al.*, 2020). Given the observed results, a temperature of ~13°C could be considered as suitable for fan mussel maintenance during disease control. The use of lower temperatures such as 8.5°C is not suitable for the species, although it has been shown to be an effective way of stopping or at least delaying mortality caused by *H. pinnae* (García-March *et al.*, 2020) for a limited period of time.

One of the major complications in captivity maintenance is that it relies on the early detection of ailing individuals. In fact, although concern about the welfare

of invertebrates is on the rise, it rarely includes (Horvath *et al.*, 2013; Carere & Mather, 2019) species other than cephalopods (Mather, 2020). But the lack of knowledge on the management and cultivation of Pinnids (Chávez-Villalba *et al.*, 2022) together with the critical situation of the species increases the need to implement welfare conditions for individuals in captivity. Given the actual situation of *P. nobilis*, the development of adequate welfare indicators for the maintenance of individuals in captivity is an urgent issue for conservation biology. Mantle retraction is currently the most commonly used indicator of fan mussel distress, although it might be a late indication of disease and reveal an already severe condition usually close to death of the individual (Prado *et al.*, 2019). Also, a slow gapping response can be understood as a deterioration of health but is complicated by the subjectivity of the observer (García-March *et al.*, 2008), differences of response related to water temperature and/or the duration of the captivity period, as individuals seem to get used to manipulation (authors pers. observation). On the contrary, CR provides an easy quantitative measure for individual monitoring in captivity as an early response and non-invasive method compared to other physiological variables, thus minimizing the stress associated with manipulation before the animal suffers further damage. CR has also been used as a biomarker of organic and inorganic contaminants, salinity, and toxicity caused by microalgae in other bivalves (Toro *et al.*, 2003; Moezzi *et al.*, 2013; Domínguez *et al.*, 2020; Gao *et al.*, 2021). Periodical control measurements could provide valuable data on individual condition and reveal early symptoms of discomfort or disease that allow the implementation of management measures, and the isolation of ailing individuals to prevent the spread of pathogens in the tank. Animals could be separated and placed in individual aquaria, which would enable the measurement of CR without further manipulation. The measurement of VO<sub>2</sub> could also provide additional information about the underlying processes such as physiological compensation (Kim *et al.*, 2001; Bayne, 2017), although this would require further handling.

**Compliance with Ethical Standards:** The authors declare that they have no conflicts of interest. The individuals were collected with permission from the competent authorities (permission reference: SGPM/BDM/AUTSPP/05/2020).

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